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Visualization of central noradrenergic neurons in thick sections by the unlabeled antibody method: A transmitter-specific Golgi image

(peroxidase antiperoxidase/dopamine-β-hydroxylase/catecholamines)

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ABSTRACT **The unlabeled peroxidase-antiperoxidase method has been used with an antiserum against rat dopamine-β-hydroxylase (DBH) to obtain a three-dimensional image of noradrenergic cell bodies and their processes in thick Vibratome sections of rat brain. This method stains DBH-positive neurons exclusively with a result similar to that of the Golgi method, which makes it possible to analyze the geometric plan of these neurons and their projections in the central nervous system. In 100-μm sections, DBH-positive axons can be followed over long distances, and the results indicate that their distribution in cerebral and cerebellar cortex is not diffuse but has a strict geometric order.**

The functional morphology of individual neurons in the brain can be described in terms of the three-dimensional geometry of their receptive surface (dendrites), the destination of their axons, and the pattern of terminal axonal branching. This has been done most successfully with the Golgi method which stains neurons in their entirety. A major advantage of this method is that it impregnates randomly only 1–2% of the total number of neurons, a feature which allows a three-dimensional analysis of nerve cells in thick sections. For an understanding of the functional role of neurons, it would be of great value if, in addition to precise morphologic knowledge of a neuronal population, information about their chemical transmitter could be obtained. With the introduction of immunohistochemical techniques into neurobiology, a powerful tool has become available to identify neurons on the basis of a specific antigenic marker (1–4). In this approach, antibodies directed against an enzyme involved in the biosynthesis of a neurotransmitter are coupled to a marker that can be visualized microscopically. Neurons that contain the enzyme can be readily identified in tissue sections that have been incubated with the labeled antiserum. The immunohistochemical staining with horseradish peroxidase-labeled antibodies is particularly attractive because the method is applicable for both light and electron microscopy (5). Although this method and its recent modification (5) have been used for the histochemical localization of tissue antigens at the electron microscopic level, their application in neurobiology has been severely hampered by the limited penetration of reactants involved in the staining procedure (6, 7).

In this report we describe an immunohistochemical method for visualizing the enzyme dopamine-β-hydroxylase [DBH; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], an antigenic marker for noradrenergic (NA) neurons in the central nervous system. This method, based on Sternberger's unlabeled antibody technique (8), results in complete staining of DBH-containing neurons in sections 100 μm thick and yields a three-dimensional image similar to that seen in Golgi preparations.

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MATERIALS AND METHODS

DBH was purified from rat adrenal glands, and antisera were raised in guinea pig (9). The specificity of this antiserum against rat DBH was demonstrated in immunodiffusion studies. Complement fixation tests showed that 25 μl of the globulin fraction diluted 1:200,000 completely fixed complement in the presence of 1.25 μg of purified enzyme.

Sprague–Dawley rats (175–225 g) were anesthetized with chloral hydrate and perfused through the ascending aorta for 5 min with ice-cold 0.15 M Na_2HPO_4, pH 7.4/2% paraformaldehyde/0.1% glutaraldehyde. Brains were promptly sliced into 5-mm slabs in either a coronal or sagittal plane. Slices glued onto chucks were sectioned at a setting of 100 μm with a Vibratome (Oxford Instruments, CA) at 5°. The entire staining procedure was carried out in 1-ml glass vials which were slowly rotated. The sections were incubated in the cold room for 24 hr with a 1:1000 dilution (30 μg/ml) of guinea pig anti-rat DBH antiserum in phosphate-buffered saline (P_i/NaCl) containing 0.4% Triton X-100. All subsequent steps were conducted at room temperature in P_i/NaCl containing 0.025% Triton X-100. Sections were washed for 3 hr with 10 changes of buffer and then incubated for 1 hr with a 1:10 dilution (2 mg/ml) of goat anti-rabbit IgG antiserum† followed by a 1-hr wash. The slices were next incubated with a 1:50 dilution of rabbit peroxidase–antiperoxidase complex (PAP) and washed again for 1 hr. After a brief wash in 50 mM Tris·HCl (pH 7.6), sections were incubated for 15 min in a solution of Tris·HCl (pH 7.6) containing 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO; 0.5 mg/ml) and 0.01% H_2O_2. The sections were washed in P_i/NaCl without Triton X-100, mounted directly on glass slides with glycerol/bicarbonate, pH 8.6, 1:1, and covered. To control for nonspecific staining, the anti-rat DBH antiserum was replaced by preimmune guinea pig serum in the first incubation step.

RESULTS

DBH-containing neurons are consistently stained in their entirety; their cell bodies, dendrites, and axons appear dark brown

Abbreviations: DBH, dopamine-β-hydroxylase (EC 1.14.17.1); NA, noradrenergic; P_i/NaCl, phosphate-buffered saline; PAP, peroxidase–antiperoxidase complex.

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† The use of a heterologous second antibody in the PAP procedure was first described by Erlandsen *et al.* (10).

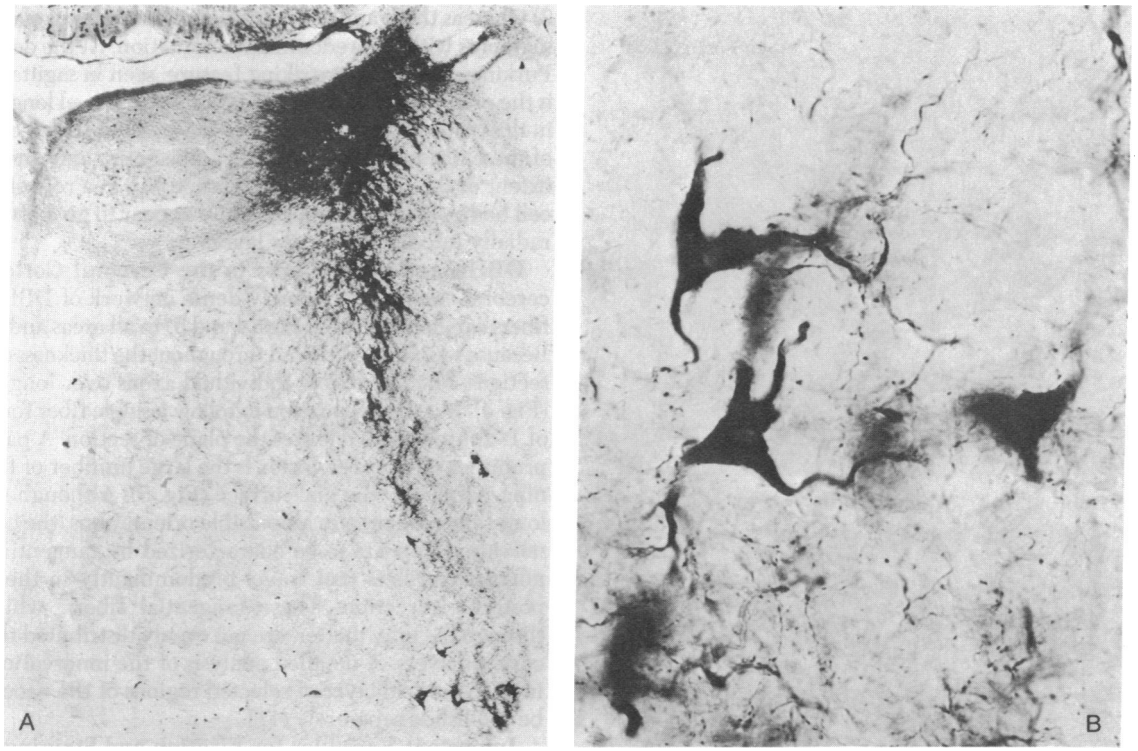

FIG. 1. (*A*) Transverse section through the pons at the level of the motor nucleus of the trigeminal nerve. The locus ceruleus is densely stained and a fiber bundle can be seen emanating from its medial aspect. Note the continuity of the nucleus proper with the row of cells extending ventrally. (×32.) (*B*) DBH-containing cells medial to the motor nucleus of the trigeminal nerve. (Transverse section; ×430.)

against a light-yellow background. Stained processess can be followed throughout the thickness of 100-μm sections. Sections incubated in preimmune serum exhibit no stained cells or processes; erythrocytes, which are occasionally present, show strong peroxidase activity.

DBH-Containing Cell Bodies. Sections stained with anti-DBH reveal the locations of DBH-containing cell bodies in the brainstem. At low magnification, the method yields a full appreciation of the spatial relationships between these neurons and landmarks such as nuclei and fiber tracts. The neurons of the locus ceruleus are intensely stained (Fig. 1*A*); in 100-μm sections, the dorsal part of the nucleus is rendered completely opaque due to the densely packed perikarya and their processes. A row of DBH-containing cells extends ventrally from the locus ceruleus into the region of the superior olivary complex. These more-ventral cells are less densely clustered, and their dendrites stand out distinctly, a feature that allows their three-dimensional arborization to be analyzed. Many of these neurons have polygonal cell bodies (20–30 μm in diameter), and tortuous dendrites issue from all sides of the soma, often with secondary and sometimes tertiary branches (Fig. 1*B*). In sections counterstained by the Nissl method, these DBH-positive cells can be shown to be medial to the motor nucleus of the trigeminal nerve.

Using this method, one can distinguish a set of DBH-containing cells with markedly different dendrite morphology (Fig. 2). These cells, located immediately rostral and slightly medial to the locus ceruleus, have ovoid cell bodies and are characterized by long, rectilinear dendrites that radiate in all directions and quickly taper close to the cell body. In some cases the axon can be seen emerging from a dendrite. Dendrites have been followed for as far as 300 μm from the soma to reveal that these cells have an extensive dendritic field, but no dendritic spines were seen on any immunoreactive cells.

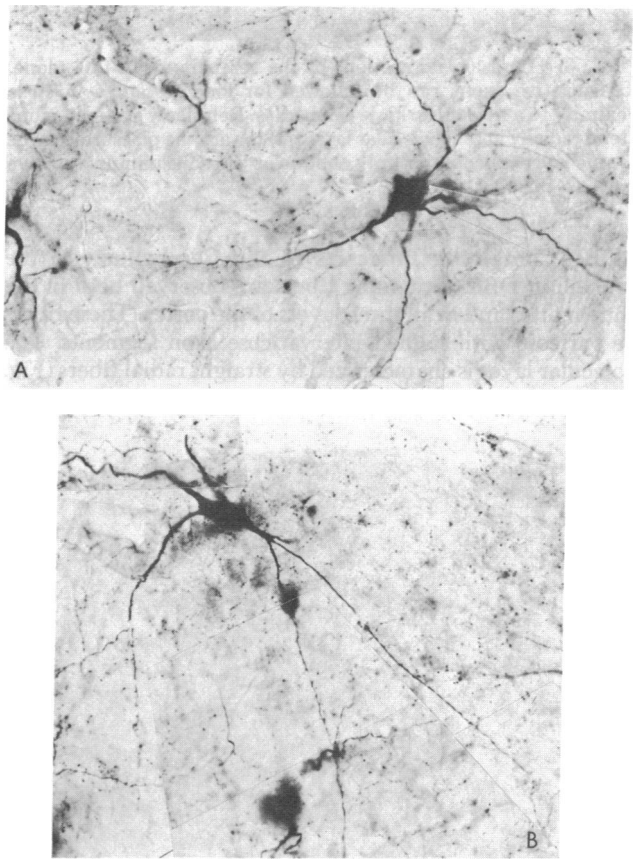

FIG. 2. Photomontage of DBH-containing neurons ventromedial to the locus ceruleus. Compare dendrites with those shown in Fig. 1*B*. (Parasagittal section; *A*, ×103.5; *B*, ×137.5.)

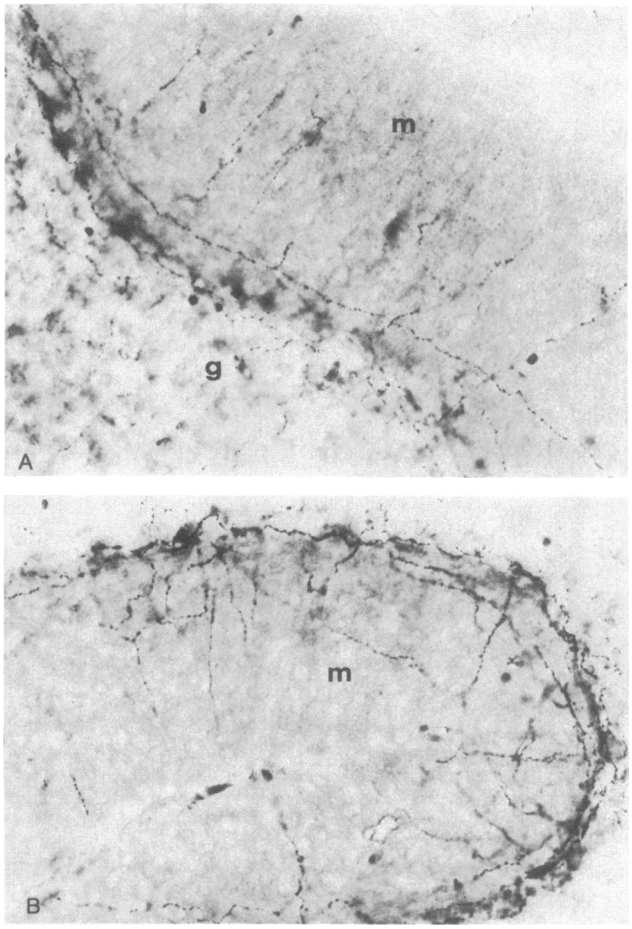

FIG. 3. Parasagittal sections of the cerebellum. In this plane, DBH-positive axons can be followed for long distances in close proximity to a row of Purkinje cell bodies. Relatively straight axons extend radially in the molecular layer (m) toward the pial surface. The granule layer (g) contains only oblique or tortuous immunoreactive axons. (×192.5.)

DBH-Containing Processes in the Cerebellum. In the cerebellum DBH-containing fibers are observed both in the white matter and in all three layers of the cortex. These fibers are varicose with stained intervaricose axon segments. The molecular layer is characterized by straight radial fibers (Fig. 3) whereas the granule cell layer contains a plexus of short axon segments with no predominant orientation. At the depth of the Purkinje cell bodies a striking feature, seen in sagittal sections, is the presence of DBH-positive axons that travel long distances in this plane of section, similar to the axons of basket cells. Some of these fibers can be traced along the entire anterior-posterior extent of the folium in close relationship to a row of Purkinje cell bodies (Fig. 3 *A* and *B*). They appear to give off branches radially into the molecular layer.

DBH-Containing Fibers in the Cerebral Cortex. In the cerebral cortex an extremely dense network of DBH-positive fibers can be visualized (Figs. 4 and 5) in all areas and all layers. Because staining is observed throughout the thickness of 100-μm sections, one can follow individual axons over long distances (Fig. 4). It is not uncommon to follow a single fiber for distances of 1–2 mm before it leaves the plane of section. A particularly prominent feature of cortex is the large number of fibers running parallel to the pial surface (Fig. 5). Although numerous long fibers are seen in all possible orientations, the cortical innervation appears to be characterized by tangential, immunoreactive fibers that travel predominantly in the anterior-posterior direction. These tangential fibers, which travel through the gray matter, are not evenly distributed through all cortical layers. A detailed analysis of the innervation pattern in each cortical layer of selected regions of the neocortex will be published separately (11).

Important Variables for Fixation and Staining. Optimal staining was achieved when rats were perfused for 5 min with ice-cold 0.15 M phosphate buffer containing 2% paraformaldehyde and 0.1% glutaraldehyde. Postfixation for 1 hr in the same fixative or perfusions for more than 10 min markedly attenuated the staining. A similar decrease in staining resulted when the glutaraldehyde concentration in the perfusate was increased (e.g., to 0.4%) while the duration of the perfusion was kept constant. Omission of glutaraldehyde resulted not only in suboptimal tissue fixation but also in fainter staining of DBH-positive fibers together with increased background staining.

Variations in the concentration of Triton X-100 in the first incubation step produced variation in the intensity of staining. No staining of DBH-containing processes was observed when Triton was omitted. Over the range in Triton concentration from 0.05 to 1%, there was an increase in staining intensity but no noticeable change in the number of processes visualized. The use of 0.4% Triton was found to yield densely stained axons and

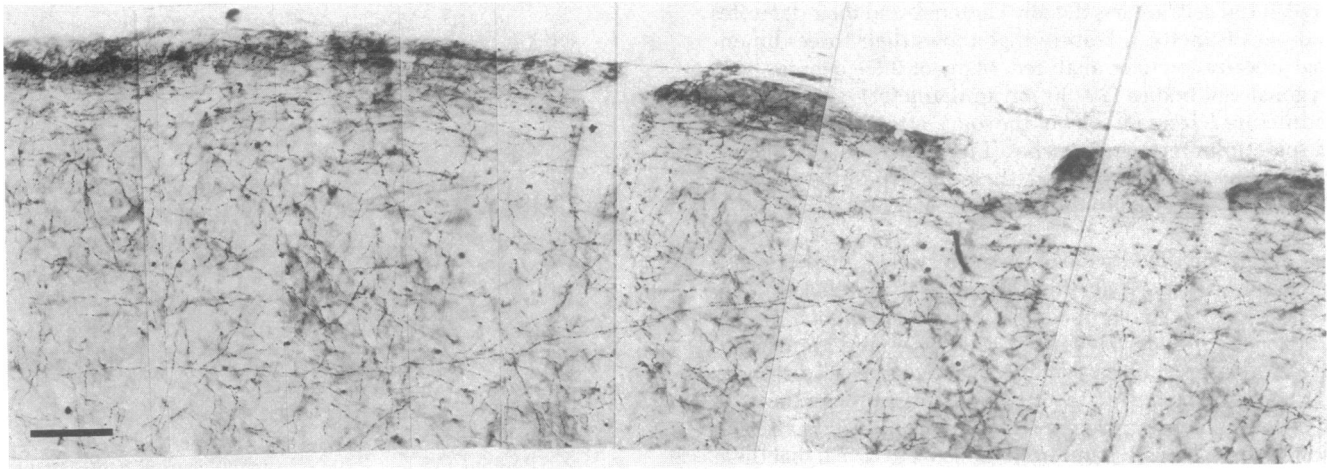

FIG. 4. Coronal section through motor cortex, showing a long DBH-positive fiber. Collage constructed from micrographs taken at different depths by focusing through the section. Pial surface is at top. Bar = 100 μm.

dendrites with minimal loss of cytologic detail. NA-cell bodies are completely stained when 0.05% Triton is used and are faintly stained even when the detergent is omitted. It should be mentioned that penetration is not dependent upon the use of a heterologous antiserum (goat anti-rabbit IgG) in the second step of the staining procedure. We did observe complete penetration of stain in thick sections in a parallel experiment in which homologous antisera were used (i.e., rabbit anti-guinea pig IgG and guinea pig PAP).

DISCUSSION

The unlabeled immunoperoxidase method allows the histochemical localization of tissue antigens at the light and electron microscopic levels. In spite of the exceedingly high sensitivity of this method, it has been reported that in thick tissue sections only the outer surfaces are stained to a depth of a few microns (6, 7). This limited penetration has allowed ultrathin sections to be prepared, with difficulty, for electron microscopic studies but prevented the use of thick sections for the study of neuronal morphology. Hence, until now, the method of choice for light microscopic immunohistochemical studies in the central nervous system has remained the fluorescent antibody method of Coons (12).

Although limited penetration of reactants in the immunohistochemical staining with the PAP technique is an often-encountered problem, the reason(s) for this are unknown. The PAP method has been criticized because it was thought that the large size of the immunochemical reagents is the primary factor that restricts penetration. The fact that we observe staining of DBH-containing fibers throughout the thickness of 100-μm sections indicates that antibodies and the PAP complex can penetrate tissue sections for at least 50 μm in less than 1 hr.

The extensive crosslinkage of tissue proteins by glutaraldehyde may partly account for the limited penetration previously reported. However, we have observed staining throughout 100-μm sections after perfusion with as much as 2% paraformaldehyde and 0.4% glutaraldehyde, whereas other investigators reported limited penetration of immunoglobulins (13) after fixation with lower concentrations of aldehydes. We cannot conclusively determine whether deficient penetration would occur in our system at higher concentrations of glutaraldehyde in the perfusate because these would inactivate antigenic sites of DBH. The role of Triton with respect to antibody penetration is difficult to evaluate because DBH is a membrane-bound protein and its immunohistochemical visualization requires the use of this detergent (2). Omission of Triton X-100 in the first incubation step leaves NA processes unstained although cell bodies in the locus ceruleus are faintly stained to a depth of 20 μm from each surface of the section. This leads us to suspect that Triton may fulfill a dual function: it exposes the membrane-bound DBH, thus making the antigenic sites of the enzyme accessible to its antibodies, and it facilitates penetration of antibodies into tissue sections.

The unlabeled antibody technique used in this study for the localization of DBH shares with the Golgi method the property of staining neurons in their entirety, which allows three-dimensional neuronal geometry to be studied in thick (100 μm) sections. This immunocytochemical method replaces the randomness of neuronal impregnation in the Golgi method with

FIG. 5. Parasagittal section of visual cortex 1 mm lateral to the midline. Photomontage is taken at the level of the splenium of the corpus callosum. Note the large number of tangential DBH-positive fibers throughout the cortex. Pial surface above; corpus callosum below. Bar = 100 μm.

a degree of selectivity that reveals solely those neurons that contain a specific biosynthetic enzyme (DBH) and thus synthesize a specific neurotransmitter (NA). Hence, with a single method, the dendritic arborization of DBH-containing neurons and their axon telodendria can be studied. The high sensitivity of the unlabeled PAP technique allows an analysis of the detailed pattern of axon termination. It is interesting to note that the immunohistochemical method does not show spines on cell bodies or dendrites of DBH-containing cells, although the Golgi–Cox method does reveal their presence on neurons in the locus ceruleus (14, 15). The spines, those regions on the surface of neurons most highly specialized for receiving afferents, contain no transmitter-synthesizing enzyme. It would be of interest to examine other neuron types with this method to determine whether it is a general principle that the spines are the only neuronal processes that are exclusively postsynaptic and contain no transmitter, especially in light of the evidence that dendrites may be both pre- and postsynaptic (16, 17).

A striking anatomic characteristic of NA axons revealed by this method is their high density in cerebral and cerebellar cortex where they travel tangentially for long distances through the gray matter. Among the speculations that have been brought forward to link the anatomical features of this projection to some functional role is the hypothesis that central NA fibers innervate intracerebral blood vessels (18, 19). Despite the high sensitivity of the PAP method and the ease with which cerebral blood vessels are seen in thick sections, we could find no evidence to support this hypothesis. In the material we have studied, the vast majority of NA fibers bear no relationship to cerebral blood vessels. Any formulation of the functional significance of the NA innervation must take into account all of the neuroanatomical features of this set of neurons. The NA projections have previously been described as having a diffuse distribution (20, 21). The present method lends itself to a more precise analysis of axon geometry and reveals that although, they are widespread, DBH-positive axons in cerebral and cerebellar cortex are not diffuse but are characterized by a highly ordered geometric pattern.

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1. Geffen, L. B., Livett, B. G. & Rush, R. A. (1969) *J. Physiol. (London)* **204**, 593–606.
2. Hartman, B. K. (1973) *J. Histochem. Cytochem.* **21**, 312–332.
3. Hökfelt, T., Fuxe, K. & Goldstein, M. (1975) *Ann N.Y. Acad. Sci.* **254**, 407–432.
4. Grzanna, R., Morrison, J. H., Coyle, J. T. & Molliver, M. E. (1977) *Neurosci. Lett.* **4**, 127–134.
5. Sternberger, L. A. (1974) *Immunocytochemistry*, (Prentice Hall, Englewood Cliffs, NJ).
6. Kawarai, Y. & Nakane, P. K. (1970) *J. Histochem. Cytochem.* **18**, 161–166.
7. Chan-Palay, V. & Palay, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4050–4054.
8. Sternberger, L. A., Hardy, P. H., Jr., Cuculis, J. H. & Meyer, H. G. (1970) *J. Histochem. Cytochem.* **18**, 315–333.
9. Grzanna, R. & Coyle, J. T. (1976) *J. Neurochem.* **27**, 1091–1096.
10. Erlandsen, S. L., Parson, J. A., Burke, J. P., Redick, J. A., Van Orden, D. E. & Van Orden, L. S. (1975) *J. Histochem. Cytochem.* **23**, 666–677.
11. Morrison, J. H., Grzanna, R., Molliver, M. E. & Coyle, J. T. (1978) *J. Comp Neurol.*, in press.
12. Coons, A. H. (1958) in *General Cytochemical Methods*, ed. Kanielli, T. F. (Academic, New York), pp. 399–422.
13. McLaughlin, B. J., Barber, R., Saito, K., Roberts, E. & Wu, J. Y. (1975) *J. Comp. Neurol.* **164**, 305–322.
14. Ramon-Moliner, E. (1974) *Cell Tissue Res.* **149**, 205–221.
15. Swanson, L. W. (1976) *Brain Res.* **110**, 39–56.
16. Shepard, G. M. (1971) *Brain Res.* **32**, 212–217.
17. Lieberman, A. R. (1974) *Z. Anat. Entwicklungsgesch.* **145**, 261–267.
18. Raichle, M. E., Hartman, B. K., Eichling, J. O. & Sharpe, L. G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3726–3730.
19. De La Torre, J. C. (1976) *Neuroscience* **1**, 455–457.
20. Freedman, R., Foote, S. L. & Bloom, F. E. (1975) *J. Comp. Neurol.* **164**, 209–232.
21. Descarries, L., Watkins, K. C. & Lapierre, Y. (1977) *Brain Res.* **133**, 197–222.